the blocked state of thin filament regulation. Because it has also been shown to stabilize the closed-state position of tropomyosin, we hypothesized that cTnI-Md is involved in the cooperativity of thin filament activation. To test this hypothesis, we generated the truncation mutant cTnI(1-167) from *R.norvegicus* wherein the entire cTnI-Md had been removed. We used passive exchange to incorporate cTnI(1-167) and FRET labeled cTnC(T13C/N51C)$_{AEDENS-DDPM}$ into left ventricular detergent skinned myocardial fibers. SDS-PAGE and Western blotting demonstrated that cTnC(T13C/N51C)$_{AEDENS-DDPM}$ and cTnI(1-167) were efficiently exchanged into skinned fibers. Intriguingly, incorporation of cTnI(1-167) resulted in biphasic Ca^{2+} dependent thin filament activation as indicated by the tightly coupled force−Ca^{2+} and N-cTnC-opening-Ca^{2+} relationships. Simultaneous force and FRET measurements showed that treatment with 1 mM orthovanadate inhibited force, reduced ensemble-averaged N-cTnC opening, and decreased the Ca^{2+}-sensitivity of activation, but did not affect the cooperativity underlying the biphasic response of N-cTnC opening to increasing Ca^{2+}. Akaike information criteria indicated that a weighted sum of two Hill equations was $>10^9$-fold superior in describing biphasic activation than a single Hill equation. Interestingly, a steady-state cooperativity model based on the concept of tropomyosin being "pinned" by cTnI (J. Mol. Biol., vol. 340, pp. 295-305) was 41-fold superior to the weighted sum of two Hill equations and suggested that blocked-state allosteric communication is severely disrupted by removal of cTnI-Md. We concluded that cTnI-Md helps facilitate allosteric communication between thin filament regulatory units in the blocked state and is therefore essential to achieving a proper contractile response to sarcomeric Ca^{2+} signals during early systole.

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Phosphorylation of Cardiac Troponin I at Tyrosine 26 Decreases Thin Filament Calcium Sensitivity

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The troponin complex is a critical molecular switch involved in transducing the calcium activating signal into contraction. Troponin I (TnI), the inhibitory subunit of the complex, is phosphorylated as a key regulatory mechanism to alter the calcium regulation of contraction. Altered cardiac contraction is a hallmark of heart failure with several studies demonstrating increased myofibrillar calcium sensitivity. Recent work has identified a novel phosphorylation of TnI at Tyr-26 that is decreased in heart failure with unknown functional effects. Similar to the location of the desensitizing TnI Ser-23/24 phosphorylation, TnI Tyr-26 is located in the unique cardiac TnI N-terminal extension. These data lead us to hypothesize that the N-terminal Tyr-26 phosphorylation of TnI decreases calcium sensitivity of the thin filament, the loss of which may contribute to the altered calcium sensitivity observed in heart failure. To assess the regulatory effects of Tyr-26 phosphorylation, we employed recombinant human cardiac TnI containing phosphate at Tyr-26 induced by treatment with a tyrosine kinase and TnI Tyr-26 phosphomimetic substitutions (Glu or Asp). The effect of TnI Tyr-26 phosphorylation on myofilament calcium sensitivity was assessed by measuring calcium binding to troponin C (TnC) in reconstituted thin filaments. Results demonstrate both Tyr-26 phosphorylation and phosphomimetics decrease calcium binding to TnC compared to filaments reconstituted with non-phosphorylated TnI. To further investigate the effects of TnI Tyr-26 phosphorylation on myofilament deactivation we measured the rate of calcium disassociation from TnC. Results demonstrate filaments containing either Tyr-26 phosphorylated TnI or phosphomimetics increase the rate of calcium dissociation from TnC. Our findings suggest that TnI Tyr-26 phosphorylation functions similarly to Ser-23/24 N-terminal phosphorylation to decrease myofilament calcium sensitivity and increase myofilament relaxation. The loss of TnI Tyr-26 phosphorylation may therefore contribute to altered cardiac contraction in heart failure.

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Effect of Amino Acid Changes in a Troponin I FHC Hotspot on Protein: Protein Binding and Calcium Sensitivity of Force Development

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Mutations in human cardiac troponin I (cTnI) have been associated with restrictive, dilated and hypertrophic cardiomyopathies. The most commonly occurring residue on cTnI that is associated with familial hypertrophic cardiomyopathy (FHC) is arginine, which is also the most common residue at which multiple mutations occur. Two FHC mutations are known to occur at arginine 204, R204C and R204H, and both are associated with poor clinical prognosis. To determine the effect of these mutations (R204C and R204H), as well as other cTnI mutations, R204P, R204Q, and R204W, calcium-force measurements and cTnI:troponin C (TnC) and cTnI:troponin T (TnT) interactions using the mammalian two-hybrid luciferase assays were utilized. All five mutations showed significant increases in calcium sensitivity of force development ranging from ΔpCa50 0.23 (R204W) to 0.35 (R204P). The mutations associated with FHC, R204C and R204H, had ΔpCa50 values of 0.28 and 0.29 respectively. The cTnI containing the R204P mutation showed the weakest interaction with TnT when compared to wild-type cTnI or the other mutants. The R204H mutation also showed significant impairment in its ability to interact with TnT, while the R204C mutation showed mild impairment when compared to wild-type cTnI. The R204C and R204P mutations showed the greatest impairment in binding to TnC. These results suggest that mutations at the same site on cTnI could affect thin filament interactions differentially, and that significant impairment in the interaction of cTnI with TnT or TnC may be enough to cause significant changes in calcium sensitivity. If the large increase in calcium sensitivity of force development observed with these mutations is associated with the poor prognosis then other R204 mutations are likely to have a poor prognosis. This research was supported by a Hellman Fellowship.

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In Vivo Analysis of Troponin C Knock-In (A8V) Mice: Evidence that TNNC1 is a Hypertrophic Cardiomyopathy Susceptibility Gene

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Recently, the *TNNC1* gene that encodes cardiac troponin C (cTnC) was found as a target for many hypertrophic cardiomyopathy (HCM) mutations in humans, eliciting alterations in the Ca^{2+} binding properties of the N-domain of cTnC. We genetically engineered knock-in mice containing the HCM-associated A8V mutation in cTnC (heterozygote = KI-TnC-A8V$^{+/-}$; homozygote = KI-TnC-A8V$^{+/+}$) in order to characterize its *in vivo*, molecular and cellular effects. ECHO revealed that at 3 months old (mo) KI-TnC-A8V$^{+/+}$ mice display increased IVRT and E/A ~1 compared to WT, suggesting diastolic dysfunction; whereas KI-TnC-A8V$^{+/-}$ showed signs of cardiac restriction at 14 mo. Histopathology of both genotyped hearts revealed papillary muscle hypertrophy, interstitial fibrosis, and myofibrilar disarray. Real-time PCR analysis at 3 mo demonstrated increases in BNP, α-MHC and β-MHC mRNA levels in the right ventricles of the KI-TnC-A8V mice (only ANP increased in the left ventricle). We identified in intact KI-TnC-A8V$^{+/-}$ and KI-TnC-A8V$^{+/+}$ cardiomyocytes: a significant decrease in the sarcomere length at several stimulation frequencies; prolonged Ca^{2+} and contractile transient kinetics at 4Hz; uncoupling between Ca^{2+} decay (delayed) and contractile (no change) transients at 6Hz; suggesting a mechanical frequency-dependent uncoupling from the Ca^{2+} transient. Furthermore, a decrease in the baseline Ca^{2+} fluorescence and in Ca^{2+} peak percentage was also detected in KI-TnC-A8V$^{+/-}$ and KI-TnC-A8V$^{+/+}$, indicating increased myofilament Ca^{2+} buffering. The calcium sensitivity of contraction in skinned fibers increased in a gene dose fashion: KI-TnC-A8V$^{+/+}$ > KI-TnC-A8V$^{+/-}$ > WT. The rate of relaxation in KI-TnC-A8V$^{+/+}$ cardiac skinned fibers investigated by flash photolysis was found increased, compared to WT. These results suggest that the A8V mutation in cTnC increases Ca^{2+} binding affinity to its N-domain eliciting changes in intracellular Ca^{2+} homeostasis and cellular mechanical function, ultimately leading to diastolic dysfunction.

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In Vitro and in Situ Structure and Function of the Cardiac Troponin C Familial Hypertrophic Cardiomyopathy-Linked Mutation, L29Q

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Familial Hypertrophic Cardiomyopathy (FHC) is characterized by severe abnormal cardiac muscle growth. The traditional view is that mutations associated with FHC result in an increase in Ca^{2+}-sensitivity of cardiac muscle contraction; however, recent studies indicate that their pathogenesis may stem from a diminished response to troponin I phosphorylation. The mutation L29Q, found in the Ca^{2+}-sensitive muscle regulatory protein, troponin C, has been tenuously linked to cardiac hypertrophy. L29Q is in the regulatory domain